Ionizing Radiation Induces Cellular Senescence of Articular Chondrocytes via Negative Regulation of SIRT1 by p38 Kinase

Eun-Hee Hong, Sang-Gu Hwang*
Division of Radiation Cancer Research, Korea Institute of Radiological and Medical Sciences, Seoul 139-706, Korea
**Corresponding author: sgh63@kirams.re.kr*

1. Introduction

Senescent cells exhibit irreversible growth arrest, large flat morphology, and up-regulated senescence-associated β-galactosidase activity at pH 6.0. Several conditions, including oncogenic stress, oxidative stress, and DNA damage are associated with cellular senescence. Massive acute DNA double-strand breaks occurring as a result of mechanical and chemical stress can be repaired, but some DNA damage persists, eventually triggering premature senescence. Since ionizing radiation directly induces DBS, it is possible that cellular senescence is activated under these conditions. The biological events in chondrocytes following irradiation are poorly understood, and limited information is available on the molecular signal transduction mechanisms of cellular senescence at present. In this study, we identify SIRT1 as a target molecule of p38 kinase and demonstrate that the interactions between p38 kinase and SIRT1 protein play an important role in the regulation of cellular senescence in response to IR.

2. Methods and Results

Individual articular chondrocytes were isolated from joint cartilage slices of 2-week-old New Zealand White rabbits by enzymatic digestion. Cells were irradiated using a ^{137}Cs-ray source (Atomic Energy of Canada, Ltd, Mississauga, Canada) at a dose rate of 3.81 Gy/min, as specified. For inhibitor or activator studies, the respective chemicals were added 1 h prior to radiation treatment. Detection of SA-β-gal activity at pH 6 was performed essentially as described by Dimri et al. In addition to ROS assay, RT-PCR, reporter gene assay, and Western blot analysis are carried out for individual experiment.

2.1 IR Induces Cellular Senescence of Primary Cultured Articular Chondrocytes

We initially assayed SA-β-gal activity, a specific cytoplasmic marker for senescent cells, after treatment with 10 Gy IR. The cytosol stained positive for senescent cells (blue) (Fig. 1A), and approximately 60% and 90% of cells displayed SA-β-gal activity at 48 h and 72 h, respectively (Fig. 1B). Consistent with SA-β-gal activity, characteristics of senescence, such as large and flat morphology, were identified in cells treated with IR in a dose-dependent manner, compared with control cells (Fig. 1C). IR additionally caused a decrease in number of chondrocytes in a concentration- and time-dependent manner (Figs 1D and 1E).

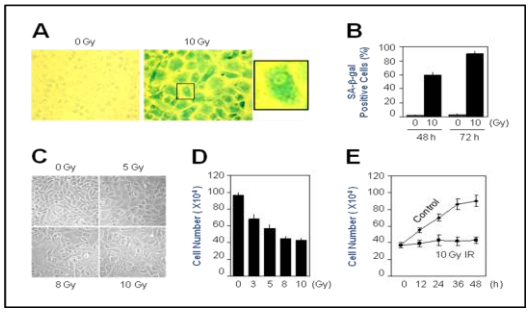

Fig. 1. IR induces cellular senescence of primary cultured articular chondrocytes.

2.2 ROS, Activated ERK and p38 MAPKs, but not JNK, are Important Mediators of IR-induced Cellular Senescence in Articular Chondrocytes

ROS levels increased significantly in a time- and dose-dependent manner in IR-exposed primary cultured articular chondrocytes, and were sustained up to 12 h after IR treatment (Fig. 2A). In contrast, ROS levels in cells pretreated with the anti-oxidants, NDGA or GSH, were comparable to those of the control group after IR treatment (Fig. 2B). IR-treated cells were positive for the SA-β-gal marker, whereas SA-β-gal was completely undetectable in cells pretreated with NDGA or GSH (Fig. 2C). However, treatment with ROS inhibitors did not lead to the recovery of senescence-specific morphological changes (Fig. 2D) or cell proliferation (Fig. 2E). These results suggest that ROS generation is necessary for induction of senescence by IR, but not maintenance of the senescence phenotype.

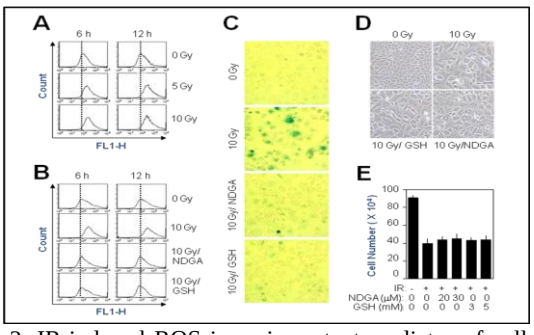

Fig. 2. IR-induced ROS is an important mediator of cellular senescence in primary cultured articular chondrocytes.

IR triggered a transient increase in the phosphorylation of ERK and JNK, leading to a peak in their activities at 30 min after treatment. Conversely, phosphorylation of p38 kinase was continuous, and

lasted up to 12 h after IR treatment (Fig. 3A). We subsequently examined whether IR-induced ROS generation accompanies MAPK activation. For this experiment, chondrocytes were preincubated with NDGA or GSH, prior to IR treatment. The compounds did not block ERK activation by IR, but inhibited p38 and JNK MAPK activation (Fig. 3B), indicating that these processes occur down-stream of ROS generation. As shown in Fig. 3C, pretreatment of cells with the p38 kinase specific inhibitor, SB203580, did not affect IR-induced ROS generation at the early time-point (90 min, upper panel), but led to the complete inhibition of ROS production at a later time-point (12 h, middle panel), with concomitant suppression of SA-β-gal activity after IR treatment (48 h, lower panel). In contrast, the ERK inhibitor, PD98059, significantly suppressed IR-induced ROS generation at the early and late time-points (upper and middle panels), as well as SA-β-gal activity (lower panel), indicating that the ERK pathway acts up-stream of ROS generation.

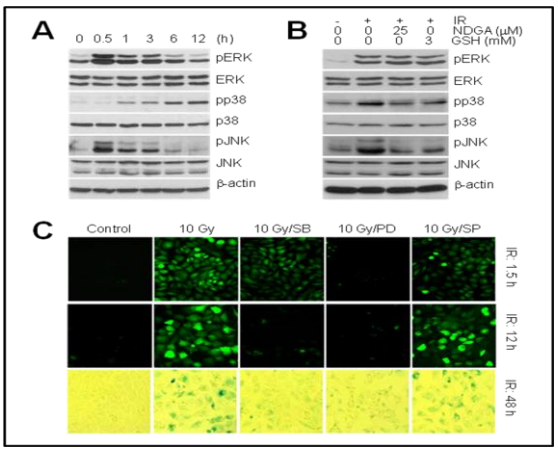

Fig. 3. . ERK and p38 kinase, but not JNK, act as pivotal mediators of ROS generation during cellular senescence of irradiated articular chondrocytes**.**

2.3 p38 Kinase down-regulates Ubiquitin-independent Post-translational SIRT1 Protein Level in IR-treated Primary Cultured Articular Chondrocytes

Phosphorylation of p38 was significantly increased at 24 h, and markedly reduced at 48 h after IR (Fig. 4A, upper panel). However, IR treatment resulted in a time-dependent reduction in the SIRT1 protein level and increased acetylation level of histone H3, a major deacetylation substrate of SIRT1. Inhibition of p38 kinase with SB203580 completely rescued IR-induced cleavage of SIRT1, whereas inhibition of ERK with PD98059 or JNK with SP600125 had no effects on the SIRT1 protein level (Fig. 4B). Specifically, overexpression of wild-type p38 synergistically abolished IR-induced degradation of SIRT1 protein, whereas SIRT1 cleavage was markedly blocked upon direct inhibition of p38 kinase with its dominant-negative form (Fig. 4C). Analysis of human SIRT1 amino sequences led to the identification of a binding site located within residues 221-261 containing the LXL motif (Fig. 4D). SIRT1 was overexpressed in chondrocytes using a retrovirus coding for wild-type SIRT1, and co-immunoprecipitation experiments performed. Immunoprecipitation of p38 kinase from lysates prepared from these cells led to increased co-precipitation of SIRT1, compared to the control group (Fig. 4E, right panel). As expected, IR suppressed complex formation between SIRT1 and p38 kinase, compared to the control group (Fig. 4E, left panel). The data suggest that p38 kinase binds to SIRT1, and regulates both protein expression and activity.

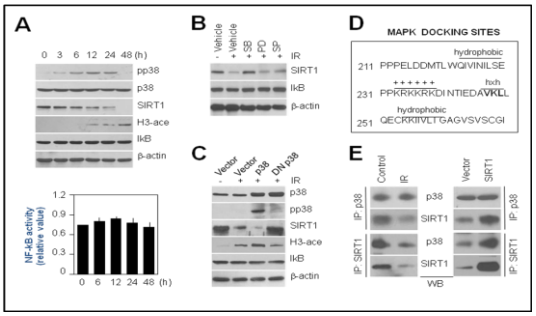

Fig. 4. p38 kinase binds to SIRT1 protein *in vivo* and negatively regulates SIRT1 expression, but not NF-kB activity in irradiated primary cultured articular chondrocytes.

3. Conclusions

In summary, induction of cellular senescence is a common response of normal cells to a DNA damaging agent, which may contribute to IR-induced normal tissue injury. Regulation of the SIRT1 level in response to IR is an important event for controlling cellular senescence. Physical interactions between p38 kinase and SIRT1 led to down-regulation of the SIRT1 protein level in IR-treated articular chondrocytes. Inhibition of senescence using SIRT1 or specific inhibitors of the p38 kinase pathway appear to be effective therapy for IR-induced normal tissue damage, and may be applied in the treatment of OA. Further studies are require to identify other molecular mechanisms promoting positive effects in maintaining healthy normal articular chondrocytes following IR exposure.

4. Refferences

1. Hristov, B., Shokek, O., and Frassica, D. A. (2007) *J. Natl. Compr. Canc. Netw.* **5,** 456-466
2. Patel, S., and DeLaney, T. F. (2008) *Cancer Control* **15,** 21-37
3. Goldwein, J. W. (1991) *Clin. Orthop. Rel. Res.* **262,** 101-107
4. Robertson, W. W. Jr., Butler, M. W., D'Angio, G. J., and Rate, W. R. (1991) *J. Pediatr. Orthop.* **11,** 284-287
5. Kroll, S. S., Woo, S. Y., Santin, A., Zietz, H., ried, H. L., Jaffe, N., and Larson, D. L. (1994) *Ann. Surg. Oncol.* **1,** 473-479